

# Determination of IgG avidity in BALB/c mice experimentally infected with *Toxocara canis*

Determinação da avidéz de IgG em camundongos BALB/c experimentalmente infectados com *Toxocara canis*

Elizandra Roselaine Schoenardie<sup>1</sup>; Carlos James Scaini<sup>2</sup>; Luciana Farias da Costa de Avila<sup>1</sup>; Rita Leal Sperotto<sup>1</sup>; Sibebe Borsuk<sup>3</sup>; Cristine Dias Pires Felicetti<sup>1</sup>; Michele Pepe<sup>3</sup>; Maria Elisabeth Aires Berne<sup>1\*</sup>

<sup>1</sup>Post-Graduate Program in Parasitology, Department of Microbiology and Parasitology, Universidade Federal de Pelotas – UFPEL, Capão do Leão, RS, Brazil

<sup>2</sup>Parasitology Laboratory, Post-Graduate Program in Health Sciences, Universidade Federal do Rio Grande – FURG, Rio Grande, RS, Brazil

<sup>3</sup>Center of Technology Development – Biotechnology, Universidade Federal de Pelotas – UFPEL, Pelotas, RS, Brazil

Received April 29, 2014

Accepted June 06, 2014

## Abstract

Toxocaríasis is a zoonotic disease in that IgM titers can remain high for long periods making difficult to determine the stage of the disease. The aim of this study is to investigate the applicability of indirect ELISA, associated with urea, to discriminate between the acute and chronic toxocaríasis. IgG avidity was evaluated in 25 BALB/c mice experimentally infected with 1000 *Toxocara canis* eggs. Blood samples were collected, and sera treated with 6 M urea and assayed by ELISA every two weeks. The percent IgG avidity was determined using the mean absorbance of sera treated with urea, divided by the mean absorbance of untreated sera. In the first 15 days post-inoculation, was observed a low percentage, between 7.25 and 27.5%, IgG avidity, characteristic of an acute infection. After 60 days of infection, all the mice showed between 31.4 and 58% IgG avidity, indicating a chronic infection.

**Keywords:** Visceral larva migrans, toxocaríasis, acute and chronic infections, ELISA.

## Resumo

A toxocaríase é uma zoonose na qual os títulos de IgM podem permanecer elevados por longos períodos, tornando difícil a determinação do estágio em que a doença se encontra. O objetivo deste estudo foi investigar a aplicabilidade de um teste indireto de ELISA, associado com ureia, para fazer a discriminação entre as fases aguda e crônica da toxocaríase. A avidéz de IgG foi avaliada em 25 camundongos BALB/c experimentalmente infectados com 1000 ovos embrionados de *Toxocara canis*. A cada duas semanas, amostras de sangue foram coletadas, o soro tratado com ureia 6M e realizado o ensaio pela técnica de ELISA. O percentual de avidéz de IgG foi determinado, usando-se a média das absorbâncias dos soros tratados com ureia dividida pela média das absorbâncias dos soros não tratados. Nos primeiros 15 dias pós-inoculação, foi observado um baixo percentual de avidéz de IgG, entre 7,25 e 27,5%, característico da fase aguda da infecção. Após 60 dias de infecção, todos apresentaram avidéz de IgG entre 31,4 e 58%, indicando a fase crônica da infecção.

**Palavras-chave:** Larva migrans visceral, toxocaríase, infecção aguda e crônica, ELISA.

## Introduction

Toxocaríasis is a zoonotic disease with worldwide distribution, caused by infection with larvae of *Toxocara canis* and *Toxocara cati*, whose definitive hosts are dogs and cats, respectively (DESPOMMIER, 2003). Children are more susceptible because of their habits of geophagy, onychophagy, poor hygienic conditions

and their larger risk of exposure to soil contaminated with parasite eggs (ALDERETE et al., 2003; SMITH et al., 2009). In tropical and developing countries, this parasite is considered a public health problem (HAYASHI et al., 2005). Clinical diagnosis is difficult due to the nonspecific signs and symptoms, making the use of immunological methods indispensable (FILLAUX; MAGNAVAL, 2013). Principally determinations of IgG, IgM and IgE are used, as hypergammaglobulinemia is an indicator of infection (DESPOMMIER, 2003). However, assessment of antibody levels alone does not determine the stage of the disease

\*Corresponding author: Maria Elisabeth Aires Berne  
 Post-Graduate Program in Parasitology, Department of Microbiology and Parasitology, Universidade Federal de Pelotas – UFPEL, Campus Universitário, s/n, CEP 96010-900, Capão do Leão, RS, Brazil  
 e-mail: bernemea@gmail.com

because, even though characteristic of acute infectious disease, IgM titers can remain high for long periods of time in *T. canis* infection (BOWMAN et al., 1987; HAVASIOVÁ-REITEROVÁ, 1995). Therefore, it is important to conduct studies to investigate IgG avidity when the serum is a reagent for both IgG and IgM (ALVARADO-ESQUIVEL et al., 2002).

Avidity is the term used to express the strength of the bond between the antibody and the multivalent antigen (HUBNER et al., 2001). The determination of IgG avidity is an important serologic marker because low and high avidity are found predominantly in early and late infection phases, respectively (FENOY et al., 2008). Studies using the ELISA immunoassay with urea, a denaturant agent that can dissociate the antigen-antibody interaction when the avidity is low (BERTOZZI et al., 1999), have been conducted to measure the IgG avidity percentage in human toxocariasis (HUBNER et al., 2001; ELEFANT et al., 2006). In experimental model, only two studies on avidity of specific IgG against TES were done (FENOY et al., 2008; KOLBEKOVÁ et al., 2011). The aim of this study is to investigate the applicability of indirect ELISA, associated with urea, to discriminate between the stages of toxocariasis infection in experimentally infected BALB/c mice.

## Materials and methods

### Parasites

Adult forms of *T. canis* were obtained by administration of pyrantel pamoate (15 mg/kg) to four- to eight-week-old dogs. Through hysterectomies performed on female worms, parasite eggs were obtained and incubated for 28 days in 2% formalin at 28 °C to allow embryonic development.

### Excretory-secretory *T. canis* antigen (TES) production

For production of TES, successive washings of the embryonated eggs, using centrifugation and phosphate-buffered saline (PBS) 0.15 M, pH 7.2, were performed. Subsequently, the eggs were treated with a solution of 5.6% sodium hypochlorite for 10 to 15 minutes. The eggs were washed again with PBS to remove the sodium hypochlorite and submitted, under aseptic conditions, to slow mechanical agitation in flasks with glass beads and RPMI 1640 medium at 37 °C (supplemented with HEPES 25 mM, Glucose 1%, penicillin 100 IU / ml, streptomycin 100 µg / ml, ofloxacin 0.4 µg / ml, and fungizone at 50 µg / ml). The larvae were then concentrated using the Baermann technique and cultured in RPMI 1640 medium at 37 °C and 5-6% CO<sub>2</sub> (DE SAVIGNY, 1975). The culture supernatant was harvested weekly, filtered through a 22-µm pore membrane (Millipore) (PAGE et al., 1991) and stored at -20 °C with protease inhibitors (PMSF - phenylmethylsulfonyl fluoride; 1 mM) (MAIZELS et al., 1991). The stored material was then mixed, concentrated by ultrafiltration (Sigma Stirred Cell - 10 kDa), dialyzed against ultrapure water (Milli-Q) at 4 °C for 24 hours, and then lyophilized. This procedure yielded TES, which was resuspended in ultrapure water and stored in aliquots at -70 °C. The TES antigen protein content was determined using a modified Bradford method (BCA kit, Pierce) (BRADFORD, 1976).

### Mice

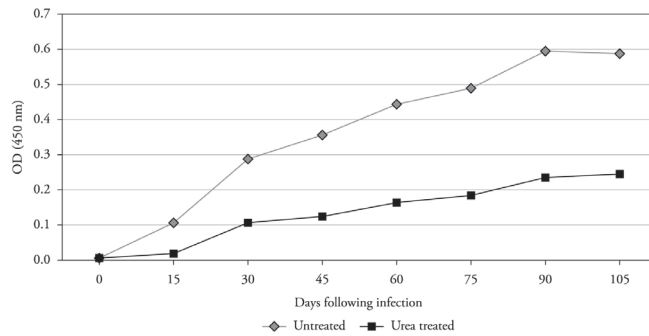
Twenty-five eight-week-old BALB/c mice were inoculated by a gastric tube with 1000 embryonated *T. canis* eggs. We evaluated the levels of anti-TES at different stages of infection. Blood samples were collected prior to inoculation (day zero) and at 15, 30, 45, 60, 75, 90, and 105 days post-inoculation. The study was approved by the Ethics in Research Committee at the Federal University of Pelotas. The experiments were carried out following the Federal Government Legislation on Animal Care.

### ELISA test

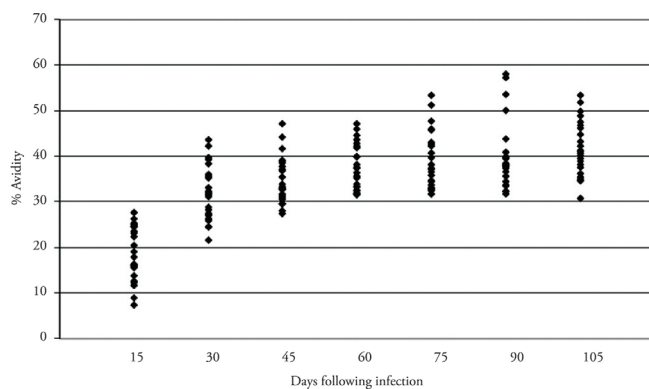
Sera from mice on the different post-inoculation days were assayed (in duplicate) by ELISA, both with and without urea. Different concentrations of urea (4 M, 5 M and 6 M), plate wash times (one, two and three minutes) and numbers of washes (one, two and three times) were tested to standardize the technique. To sensitize the plate, we used TES antigen (1 µg/ml) in carbonate/bicarbonate buffer, pH 9.6 for three hours at 37 °C, followed by blocking for one hour with PBS 2% casein and adding the sera of the inoculated animals (1:50) in PBS-T for 45 minutes. Then, the plate was washed with PBS-T either containing urea or not containing urea, and the conjugate was added (anti-mouse IgG linked to peroxidase - 1:2000). The substrate orthophenylenediamine (OPD) was used at a concentration of 0.4 mg/ml in citrate-phosphate buffer (pH 4.0) with hydrogen peroxide 0.1%. Readings were performed on a spectrophotometer (450 nm). The ELISA avidity test was performed according to Fenoy et al. (2008), with modifications. The calculation of the IgG avidity was determined using the arithmetic mean absorbance obtained in sera treated with urea divided by the mean absorbance obtained from untreated sera and was expressed as a percentage (BERTOZZI et al., 1999). Statistical analysis was performed using Statistix 8. To compare the absorbance of sera (treated or untreated with urea) on different days after inoculation, we used variance analysis with repeated measurements and compared the averages using the "Least Significant Difference" (LSD).

## Results and discussion

To assess the avidity of IgG against TES, the ELISA was first standardized with different concentrations of urea, obtaining the best result with a concentration of 6 M and a two-minute plate wash. IgG levels in the sera after treatment with urea were reduced significantly ( $P < 0.01$ ) in all periods analyzed (Figure 1). When analyzing the percentages of IgG, in each of the twenty-five BALB/c mice, we observed a percentage drop (to low avidity) at 15 days post infection (7.25 to 27.5%), suggesting that acute infection had begun in this period. An intermediate avidity for IgG was observed until 45 days post-inoculation (between 27.5 and 31.4%), and at 60 days post-infection, all the animals presented an IgG avidity index of between 31.4 and 58% (Figure 2), which characterizes the chronic phase of toxocariasis (FENOY et al., 2008). The result of the present study are according to Fenoy et al. (2008) that found high-avidity of IgG antibodies (over 50%) 60 days post-infection



**Figure 1.** Mean absorbance of IgG in serum of mice experimentally infected with *T. canis*, untreated or treated with 6 M urea. Treated and untreated sera in the graph differ significantly ( $P < 0.01$ ) for all post-inoculation days evaluated.



**Figure 2.** Percentage of avidity of anti-TES IgG using Indirect ELISA in 25 experimentally infected mice with 1000 eggs each of *T. canis* on different days post-inoculation.

of BALB/c with 1000 *T. canis* eggs. However, another study using BALB/c mice reported high-avidity of IgG antibodies at only 30 days post-infection of 1000 larvae (KOLBEKOVÁ et al., 2011).

Similar data were obtained from serological analysis of 1376 individuals in the Czech Republic, where an avidity index higher than 40% was presented, characterizing chronic infection (HUBNER et al., 2001). Although mice are the most widely used experimental models in the study of toxocariasis, they may exhibit some differences in the avidity of IgG antibodies for TES. This difference was observed in *Toxoplasma gondii* infection, in both humans and sheep, where chronic infection in humans was established when the percentage of IgG avidity was higher than 40%, which occurred after five months of infection (BERTOZZI et al., 1999). In sheep, the percentage of avidity was greater than 35%, which occurred after the fourth week of infection (SAGER et al., 2003).

The evaluation of IgG avidity has been widely used in the routine diagnosis of various infectious diseases, not only toxoplasmosis (LAPPALAINEN; HEDMAN, 2004; RAHBARI et al., 2012), but also rubella (REIS et al., 2004), varicella (ONO et al., 2004; KNEITZ et al., 2004) and cytomegalovirus (BONALUMI et al., 2011), been very useful for assessing seroconversion during gestation (BOBIC et al., 2009). The avidity of IgG antibody against TES

is potentially useful for immunodiagnosics of toxocariasis and should be included in the complementary diagnosis when IgM levels remain high throughout the infection. The present study demonstrated that the ELISA enzyme immunoassay with urea is an alternative for discriminating the stages of toxocariasis infections in BALB/c mice. The difficulty in diagnosing an active infection by *T. canis* is a serious problem, and many studies are still needed.

## References

- Alderete JMS, Jacob CMA, Pastorino AC, Elefant GR, Castro APM, Fomin ABE, et al. Prevalence of *Toxocara* infection in schoolchildren from the Butantã region, São Paulo, Brazil. *Mem Inst Oswaldo Cruz* 2003; 98(5): 593-597. PMID:12973524. <http://dx.doi.org/10.1590/S0074-02762003000500002>
- Alvarado-Esquivel C, Sethi S, Janitschke K, Hahn H, Liesenfeld O. Comparison of two commercially available avidity tests for *Toxoplasma*-specific IgG antibodies. *Arch Med Res* 2002; 33(6): 520-523. [http://dx.doi.org/10.1016/S0188-4409\(02\)00411-3](http://dx.doi.org/10.1016/S0188-4409(02)00411-3)
- Bertozi LC, Suzuki LA, Rossi CL. Serological diagnosis of toxoplasmosis: usefulness of IgA detection and IgG to avidity determination in a patient with a persistent IgM antibody response to *Toxoplasma gondii*. *Rev Inst Med Trop S Paulo* 1999; 41(3): 175-177. PMID:10529837. <http://dx.doi.org/10.1590/S0036-46651999000300008>
- Bobic B, Klun I, Vujanic M, Nikolic A, Ivovic V, Zivkovic T, et al. Comparative evaluation of three commercial *Toxoplasma*-specific IgG antibody avidity tests and significance in different clinical settings. *J Med Microbiol* 2009; 58(3): 358-364. Available from: <http://jmm.sgmjournals.org/content/58/3/358.long> PMID:19208887. <http://dx.doi.org/10.1099/jmm.0.006668-0>
- Bonalumi S, Trapanese A, Santamaria A, D'Emidio L, Mobili L. Cytomegalovirus infection in pregnancy: review of the literature. *J Prenat Med* 2011; 5(1): 1-8. Available from: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3279147> PMID:22439067 PMCid:PMC3279147.
- Bowman DD, Mika-Grieve M, Grieve RB. Circulating excretory-secretory antigen levels and specific antibody responses in mice infected with *Toxocara canis*. *Am J Trop Med Hyg* 1987; 36(1): 75-82. Available from: <http://www.ajtmh.org/content/36/1/75.long> PMID:3812886.
- Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72(1,2): 248-254.
- De Savigny DH. In vitro maintenance of *Toxocara canis* larvae and a simple method for the production of *Toxocara* ES antigens for use in serodiagnostic tests for visceral larva migrans. *J Parasitol* 1975; 61(4): 781-782. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/1165568> <http://dx.doi.org/10.2307/3279492>
- Despommier D. Toxocariasis: clinical aspects, epidemiology, medical ecology, and molecular aspects. *Clin Microbiol Rev* 2003; 16(2): 265-272. Available from: <http://cmr.asm.org/content/16/2/265.full> PMID:12692098 PMCid:PMC153144. <http://dx.doi.org/10.1128/CMR.16.2.265-272.2003>
- Elefant GR, Shimizu SH, Sanchez MCA, Abe Jacob CM, Ferreira AW. A Serological Follow-up of Toxocariasis patients after chemotherapy based on the detection of IgG, IgA, and IgE antibodies by Enzyme-Linked Immunosorbent Assay. *J Clin Lab Anal* 2006; 20(4): 164-172. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16874812> PMID:16874812. <http://dx.doi.org/10.1002/jcla.20126>

- Fenoy S, Rodero M, Pons E, Aguila C, Cuéllar C. Follow-up of antibody avidity in BALB/c mice infected with *Toxocara canis*. *Parasitology* 2008; 135(6): 725-733. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18413003> PMID:18413003. <http://dx.doi.org/10.1017/S0031182008004368>
- Fillaux J, Magnaval JF. Laboratory diagnosis of human toxocariasis. *Vet Parasitol* 2013; 193(4): 327-336. PMID:23318165. <http://dx.doi.org/10.1016/j.vetpar.2012.12.028>
- Havasiová-Reiterová K, Tomasovicová O, Dubinský P. Effect of various doses of infective *Toxocara canis* and *Toxocara cati* eggs on the humoral response and distribution of larvae in mice. *Parasitol Res* 1995; 81(1): 13-17. Available from: <http://link.springer.com/article/10.1007%2FBF00932411> PMID:7724509. <http://dx.doi.org/10.1007/BF00932411>
- Hayashi E, Tuda J, Imada M, Akao N, Fujita K. The high prevalence of asymptomatic *Toxocara* infection among schoolchildren in Manado, Indonesia. *Southeast Asian J Trop Med Public Health* 2005; 36(6): 1399-1406. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16610641> PMID:16610641.
- Hubner J, Uhlíková M, Leissová M. Diagnosis of the early phase of larval toxocariasis using IgG avidity. *Epidemiol Mikrobiol Immunol* 2001; 50(2): 67-70. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11329729> PMID:11329729.
- Kneitz RH, Schubert J, Tollmann F, Zens W, Hedman K, Weissbrich B. A new method for determination of varicella-zoster virus immunoglobulin G avidity in serum and cerebrospinal fluid. *BMC Infect Dis* 2004; 4:33. Available from: <http://www.biomedcentral.com/1471-2334/4/33>
- Kolbeková P, Větvička D, Svoboda J, Skírnisson K, Leissová M, Syrůček M, et al. *Toxocara canis* larvae re-infecting BALB/c mice exhibit accelerated speed of migration to the host CNS. *Parasitol Res* 2011; 109(5): 1267-1278. Available from: <http://link.springer.com/article/10.1007%2Fs00436-011-2371-y> PMID:21537988. <http://dx.doi.org/10.1007/s00436-011-2371-y>
- Lappalainen M, Hedman K. Serodiagnosis of toxoplasmosis: The impact of measurement of IgG avidity. *Ann Ist Super Sanità* 2004; 40(1): 81-88. Available from: <http://www.iss.it/publ/anna/2004/1/40181.pdf> PMID:15269456.
- Maizels RM, Blaxter ML, Robertson BD, Selkirk ME. *Parasite antigens, parasite genes: a laboratory manual for molecular parasitology*. Cambridge: Cambridge University Press; 1991.
- Ono E, Lafer MM, Weckx LY, Granato C, Moraes-Pintos M. A simple and cheaper in house varicella zoster virus antibody indirect ELISA. *Rev Inst Med Trop S Paulo* 2004; 46(3): 165-168. PMID:15286822. <http://dx.doi.org/10.1590/S0036-46652004000300008>
- Page AP, Richards DT, Lewis JW, Omar HM, Maizels RM. Comparison of isolates and species of *Toxocara canis* and *Toxocara* by biosynthetic labelling of somatic and ES proteins from infective larvae. *Parasitology* 1991; 103(3): 451-464. Available from: [http://journals.cambridge.org/download.php?file=%2FPAR%2FPAR103\\_03%2FS0031182000059977a.pdf&code=f43dacc3db867ce30efbe7a12d05dd39](http://journals.cambridge.org/download.php?file=%2FPAR%2FPAR103_03%2FS0031182000059977a.pdf&code=f43dacc3db867ce30efbe7a12d05dd39) PMID:1780182. <http://dx.doi.org/10.1017/S0031182000059977>
- Rahbari AH, Keshavarz H, Shojaee S, Mohebbi M, Rezaeian M. IgG avidity ELISA Test for Diagnosis of Acute Toxoplasmosis in Humans. *Korean J Parasitol* 2012; 50(2): 99-102. Available from: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3375462/> PMID:22711919 PMID:PMC3375462. <http://dx.doi.org/10.3347/kjp.2012.50.2.99>
- Reis MM, Tessaro MM, Silva JC, Giordano AS, d' Azevedo PA. Avidity of IgG for Rubella: an evaluation of the need for implementation at the materno-infantil Presidente Vargas Hospital in Porto Alegre, Rio Grande do Sul, Brazil. *Braz J Infect Dis* 2004; 8(3): 249-254. PMID:15476057. <http://dx.doi.org/10.1590/S1413-86702004000300009>
- Sager H, Gloor M, Tenter A, Maley S, Hässig M, Gottstein B. Immunodiagnosis of primary *Toxoplasma gondii* infection in sheep by the use of a P30 IgG avidity ELISA. *Parasitol Res* 2003; 91(2): 171-174. Available from: <http://link.springer.com/article/10.1007%2Fs00436-003-0964-9> PMID:12923635. <http://dx.doi.org/10.1007/s00436-003-0964-9>
- Smith H, Holland C, Taylor M, Magnaval JF, Schantz P, Maizels RM. How common is human toxocariasis? Towards standardizing our knowledge. *Trends Parasitol* 2009; 25(4): 182-188. PMID:19269251. <http://dx.doi.org/10.1016/j.pt.2009.01.006>